UNIVERSITY OF OKLAHOMA MEDICAL CENTER

THE PULMONARY ULTRASTRUCTURE IN SEPTIC SHOCK

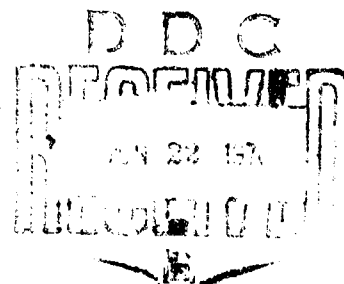J. J. COALSON, L. B. HINSHAW, AND C. A. GUENTER

Technical Report No. 20
University of Oklahoma Medical Center THEMIS Contract

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MEDICAL CENTER RESEARCH AND DEVELOPMENT OFFICE
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INTRODUCTION

Kuida and others (Kuida et al., 1961) observed that the primate lung was markedly affected by the administration of endotoxin. Reported pulmonary pathological alterations have varied due to prominent species differences observed in the reaction to endotoxin. Differences have also been observed between experiments utilizing a single injection of endotoxin and those involving the generalized Shwartzman phenomenon (Wong, 1962; McKay et al., 1966; Horn and Collins, 1968b). Abnormal pulmonary hemodynamics and morphology following a single injection of endotoxin in dogs have been reported (Kuida et al., 1961; Harrison et al., 1969). Pulmonary changes after endotoxin in rats or rabbits include platelet agglutination within pulmonary capillaries (Stetson, 1951; McKay et al., 1966), rupture of capillary walls (DePalma et al., 1967), fibrin deposition (McKay et al., 1966; DePalma et al., 1967), clumps of polymorphonuclear leukocytes (Stetson, 1951; DePalma et al., 1967; Horn and Collins, 1968b), and polymorphonuclear leukocyte fragmentation within the lung capillaries (McKay et al., 1966; Horn and Collins, 1968b). These results, however, were obtained in animals other than primates and in several of the studies (Wong, 1962; McKay et al., 1966; Horn and Collins, 1968b) more than one injection of endotoxin was used and may have elicited the generalized Shwartzman phenomenon. Previous work (Harrison et al., 1969) utilizing a single injection of endotoxin in dogs demonstrated leukocytic sequestration and fragmentation and loss of cytoplasmic specific granules in polymorphonuclear leukocytes with an apparent accumulation of glycogen. A single investigation of pulmonary ultrastructural changes has been carried out in the monkey administered endotoxin (McKay et al., 1967). In their study, at 15 minutes, leukocytes were observed distending the pulmonary

capillaries; at one hour, a decrease of polymorphonuclear leukocytes was reported and the appearance of fibrin strands was noted. These authors emphasized that intravascular coagulation, sequestration and destruction of polymorphonuclear leukocytes, and platelet agglutination were commonly observed in the primate following endotoxin injection. The major purpose of the current study was to extend and re-evaluate the previous investigation on the primate administered endotoxin. Waisbren (1964) recently criticized the animal shock model in which endotoxin was used to simulate septic shock in man. Pulmonary functional abnormalities reported in primates administered live E. coli organisms (Guenter et al., 1969a; Guenter et al., 1969b) have included among other changes, hyperventilation, increased physiological dead space, increased alveolo-arterial oxygen gradients, decreased compliance of the lungs and increased surface tension of lung extracts. The ultrastructural morphological effects of live organisms in the lung of the primate have not been previously described. The second purpose of the present investigation was to utilize an animal model more closely approximating the clinical entity of septic shock by including a series of experiments with intravenously injected live E. coli organisms.

MATERIALS AND METHODS

Ten adult rhesus monkeys of both sexes, ranging in weight from 4.6 to 10 kgs., were selected for this study. In each instance the animal was injected with 20 to 30 mg/kg of pentobarbital intravenously. Surgery was then performed to expose the femoral artery and vein. A polyethylene catheter was introduced into the femoral vein and advanced to the inferior vena cava. Anticoagulants were not used. A teflon needle was introduced into the femoral artery. The systemic arterial pressure was continuously measured. The animals

were intermittently ventilated with positive pressure through t the study to prevent atelectasis. Six animals were given infusions of 6 mg/kg $E.$ $\underline{coli}$ endotoxin (Difco) and four were given 4×10^9 organisms/kg live $\underline{E}.$ $\underline{coli}$, prepared as previously described (Hinshaw et al., 1968). Two animals were sacrificed at 15 minutes, two at one hour, and two were sacrificed at 4 hours after the administration of endotoxin. Two animals were sacrificed at one hour and two were sacrificed at four hours after the administration of the live organisms. Control monkeys had been studied during the previous parallel study (Guenter et al., 1969a). Sacrifice was accomplished by injection of massive doses of pentobarbital and the lungs were immediately removed. To ascertain the diffuseness of the pulmonary lesions in each animal, tissues were sampled from 3 lobes: the right upper lobe, right lower lobe, and lingula in one animal and the left upper lobe, left lower lobe, and right middle lobe in the other. Thus in each pair of animals, observations were obtained from 6 areas of lung. Specimens for light and electron microscopic studies were obtained at the above mentioned time intervals. For light microscopic tissue studies, Carnoy's and Bouin's were the fixatives employed. The tissue was embedded in Paraplast, and sections were stained with hematoxylin and eosin, Periodic acid Schiff with diastase controls and Weigert's hematoxylin fibrin stain. The samples for ultrastructural study were fixed in Zetterqvist's fixative, dehydrated in alcohol and embedded in Epon 812 and Maraglas. Thin sections were obtained and stained with acidified uranyl acetate and one of the following lead stains, Karnovsky's lead stain B (1961) or Reynold's lead citrate (1963). The sections were examined with an RCA EMU-3F electron microscope with appropriate fields photographed.

RESULTS

A. Physiologic Studies

The average mean systemic arterial pressure in all animals was 120 mm Hg (S.E.=9.9 mm Hg) prior to injection of E. coli or endotoxin and decreased at least 40% prior to the time of sacrifice (Figure 1).

B. Pathologic Studies

(1) Light microscopic observations

At 15 minutes, endotoxin-treated lung samples showed rounding of the alveolar spaces, focal pulmonary edema, patchy atelectasis, and ectasia with engorgement of the vasculature with red blood cells and neutrophilic leukocytes (Figure 2). No evidence of fibrin thrombi was observed.

At one hour in both endotoxin and live organism specimens, the capillaries were markedly ectatic and filled with leukocytes. More of the pulmonary parenchyma showed eviden of pulmonary edema; however, this was not noted to be diffuse throughout the lung tissue. Concomitant with the increase of pulmonary edema, the atelectasis was more pronounced. Focal intra-alveolar hemorrhage was seen at this time.

At four hours in both endotoxin and live organism specimens, the changes described above were seen; however, the severity of the findings were accentuated and were more extensively noted in the lungs (Figure 3). None of the material examined showed bilateral diffuse pulmonary edema.

Special stains for fibrin did not reveal evidence of fibrin thrombi in any of the material examined. Periodic acid-Schiff with diastase controls for glycogen showed prominent glycogen demonstrability in the leukocytes of the four-hour specimens of both the endotoxin and the live organism-treated animals.

(2) Electron microscopic studies

Ultrastructurally, endotoxin-treated lung parenchyma at 15 minutes was characterized by ectasia and plugging with neutrophilic leukocytes in the pulmonary capillaries (Figure 4). Fragmenting neutrophils were seen intravascularly (Figure 5). Some loss of the specific granules of the leukocytes was demonstrable (Figures 4,5). The alveolar type I epithelium (membranous pneumocytes) was edematous, and in focal areas disrupted. The alveolar type II cells (granular pneumocytes) were essentially normal (Figure 5). Occasional blocks examined showed an alveolar space transudate; however, this was an infrequent observation. The capillary endothelium maintained its structural continuity, and no evidence of disruption or alteration was seen except where the leukocytes were sticking to the endothelial walls. At these points of attachment, the endothelium showed a fuzzy indistinct border (Figure 4).

At one hour, both endotoxin and live organism-treated animals showed alveolar type I cytoplasmic edema and focal disruption, unaltered alveolar type II cells, and mild to moderate perivascular space edema (Figure 6). Significant changes from the 15 minute samples were the degranulation of the leukocytes, leukocytic fragmentation, and the appearance of glycogen deposits within the leukocytic cytoplasm (Figures 6,7,8). Occasional platelets were seen. Increased free lamellar material was seen within alveolar spaces (Figure 6), and sites of pulmonary edema were more frequently noted. Again, the capillary endothelium was essentially intact except for those sites of leukocytic attachment (Figures 6,7,8). An increase of pinocytotic vesicles and large vacuoles was evident within the endothelium (Figures 6, 7,8).

At four hours the alveolar type I epithelium showed similar changes in 15-minute and 1-hour samples (Figure 9). Some of the alveolar type II

cells were characterized by early degenerative changes consisting of fatty
change of the cytosomes, blunting of the microvilli and moderate rarefaction
of the cytoplasm. The edematous changes were again noted in all the peri-
vascular space (Figures 9,10,11,12,13,14). Pulmonary edema and lamellar
material were frequently observed. Intravascularly, free granules, frag-
menting leukocytes, degranulated leukocytes with glycogen deposits and a
few platelets were characteristically present (Figures 9,10,11,12,13,14).
Endothelial damage was still not apparent except in those areas of leu-
kocyte sticking (Figures 9,10,11,12,13,14). Atelectasis, with the collapse
of alveolar walls, was noted. No differences were discerned between the
endotoxin and live organism-treated tissues.

DISCUSSION

The present study demonstrated sequestration and fragmentation of
neutrophils in pulmonary capillaries within 15 minutes following injection
of endotoxin or E. coli organisms. This obstructive action could con-
ceivably account for results previously reported by Guenter, Fiorica and
Hinshaw (1969a) in which endotoxin or E. coli organism injection in mon-
keys resulted in hyperventilation, and increased physiological dead space.
It is conceivable that the mechanical presence of large numbers of white
cells in pulmonary capillaries may result in pulmonary hypertension, open-
ing up of previously closed pulmonary vascular channels, and development
of pulmonary arteriovenous shunts, thus carrying elevated alveolo-arterial
oxygen gradients as previously reported (Guenter et al., 1969a). It is
also known that histamine injection can simulate the pulmonary vascular
changes found in endotoxin-treated animals (Gilbert et al., 1953; Kuida
et al.,1958; Kuida et al., 1961). A diffuse obstructive plugging by
polymorphonuclear leukocytes throughout the pulmonary capillaries may

elicit histamine release from mast cells in the lung leading to vasodila-
tation and increased capillary permeability.

McKay and others (McKay et al., 1967) noted that leukocytes were ob-
served distending the pulmonary capillary bed at 15 minutes following in-
jection of endotoxin in monkeys. They further observed a subsequent de-
crease of polymorphonuclear leukocytes at one hour with the appearance of
fibrin strands. A marked difference in results, however, is noted between
the findings of the present study and those of McKay (McKay et al., 1967),
in that in no tissue examined was there evidence of fibrin strands. The
reason for the discrepency between the experiments of McKay and others
(McKay et al., 1967) and the current investigation is not understood. It
is conceivable that their experiments involved use of excessive injections
of endotoxin or that interfering impurities were present in the endotoxin
moiety. Experiments carried out in this laboratory have shown that the
lethality effects and hemodynamic alterations in primates given endotoxin
are similar regardless of whether the animals are heavily heparinized or when
no heparin is administered in any experiment. In addition, past experiments
in this laboratory with the use of light microscopy have not demonstrated
the appearance of fibrin thrombi in the primate administered endotoxin.

The present study revealed focal areas of perivascular space edema in
all tissues examined following injections of both E. coli live organisms
or endotoxin. Focal pulmonary edema was found to be accentuated four hours
following injection. Previously reported altered ventilation perfusion re-
lationships, decrease in pulmonary compliance, and altered surface tension
properties of lung extracts may be explained on the basis of widespread but
focal perivascular edema as demonstrated in the ultrastructural studies of
the present investigation. Perivascular space edema has been shown to

elevate intrapulmonary venous pressure (West, 1965). Using staphlococcal enterotoxin B treated monkeys, Finegold (1967) observed an increase of fluid within the perivascular space concomitant with endothelial damage. Increased pinocytotic vesiculation has been noted in electron microscopic studies in which there is an increased perfusate (Coalson et al., 1967) or increased absorption of fluid (Leeson and Leeson, 1964). The quantity of vesicles and their size are helpful in evaluating the increase of endothelial fluid transport (Schulz, 1959). Abundant pinocytotic vesicles, including many of large size, were observed in the lung tissue studied in the present report. Larger cytoplasmic vacuoles were also demonstrated. Changes in surfactant due to the changes in the alveolar type II cell seem unlikely at least where the shock is only of this duration. The alveolar type II cells were only mildly damaged in the 4-hour samples of endotoxin and live organism lung samples, and damaged cells were found usually in an area of focal pulmonary edema.

In the present study, endothelial smudging considered indicative of loss of structural integrity was noted only at sites where the leukocytes were sticking to the capillary walls. Whether or not released lysosomal enzymes initiated the vascular alteration of stickiness has not been elucidated. It is interesting to note that adrenal corticoids stabilize lysosomes (Weissmann and Dingle, 1961; Weissmann and Thomas, 1963) and also prevent endothelial sticking (Allison et al., 1955; Grant et al., 1962). The question of whether these sites are points at which possible permeability leaks could occur is another unresolved point. Increased capillary permeability can occur before actual sticking of the polymorphonuclear leukocytes is observed (Allison and Lancaster, 1959). In the present studies,

It was observed that the polymorphonuclear leukocytes showed no evidence of active emigration. With the exception of one previous study in which pulmonary capillary rupture was found (DePalma et al., 1967), the finding of intact endothelial cytoplasm has been consistently described (McKay et al., 1966; McKay et al., 1967; Horn and Collins, 1968b; Harrison et al., 1969). Other than for the smudging noted at sites of endothelial-polymorphonuclear leukocytes sticking, these results are consistent with the above reports.

In regard to the role of lysosomes in endotoxin shock, a previous investigation (Martini, 1959) has shown that at two hours after endotoxin an increase of free catheptic activity is present in liver and muscle homogenates. Using endotoxin-treated liver homogenates, Weissmann and Thomas (1962) reported a release of cathepsin and B-glucuronidase from the lysosomal fraction into the non-sedimentable fraction of the homogenates. Increased blood levels of B-glucuronidase and acid phosphatase with a concomitant depletion of these enzymes in the liver homogenate lysosomal fraction after endotoxin injection were reported by Janoff (Janoff et al., 1962).

The specific granules of the neutrophils are felt to be lysosomes due to their morphologic composition, enzymatic contents, and functional activities (Cohn and Hirsch, 1960a; Cohn and Hirsch, 1960b). It has been shown that neutrophilic granules may function to damage cells in Shwartzman and Arthus reactions (Thomas, 1964) and can elicit intravascular coagulation when given to endotoxin-treated animals (Horn and Collins, 1968a). Whether or not released neutrophilic lysosomes damage the pulmonary capillary endothelium after a single endotoxin injection has not been clarified. Our results would indicate that the lysosomal granules do not visibly affect the structural continuity of the capillary endothelium. Degranulation of the

leukocytes has long been known to result during phagocytosis (Robineaux and Frederick, 1955; Hirsch and Cohn, 1960; Sbarra et al., 1961). This morphologic finding has been confirmed by biochemical studies in which there is a transfer of the hydrolytic enzymes within the specific granular fraction to the supernatant fraction (Cohn and Hirsch, 1960b). In the present study, the use of live organisms could readily explain the resultant degranulation and disruption of t..e polymorphonuclear leukocytes on the basis of an active phagocytic function, but the actual decrease of specific granules observed in endotoxin-treated animals cannot be as easily explained. There has been some attempt to explain the action of endotoxin on polymorphonuclear leukocytes on the basis of "sham phagocytosis" (Weissmann and Thomas, 1964). These workers feel that endotoxin does not have a direct action on the lysosomes, but hypothesize that an increase in glycolysis and oxygen consumption is induced by endotoxin, lowering the intracellular pH, thus rendering the lysosomal membranes more permeable to the enclosed enzymatic contents. Subsequent lysis and release of the granules could then occur with a resultant solubilization of the enzymes.

In the previous morphologic studies in which endotoxin was used, several of the investigators have not emphasized this finding of loss of the specific granules in the leukocytes, although their electron micrographs showed this change. In the present study, the effects of endotoxin and live organisms characteristically elicited degranulation of the specific granules of the polymorphonuclear leukocytes.

The effects of the disintegration of polymorphonuclear leukocytes and the eventual breakdown of their lysosomes in the pulmonary capillaries have not been explored and remain to be answered. The findings in this study

would indicate that the use of endotoxin or live organisms elicit markedly similar morphologic lesions. It is further noted that in contrast to a previous report (McKay et al., 1967) in the primate administered endotoxin, this study did not reveal any evidence of intravascular fibrin accumulation. The widespread accumulation of intravascular polymorphonuclear leukocytes and perivascular space edema may explain some of the physiologic functional derangements known to occur in monkeys in shock.

SUMMARY

The ultrastructural alterations in the lungs of the monkey after intravenously administered let'al injections of live E. coli organisms or endotoxin are markedly similar. Edema of the perivascular space was seen in all lung tissues examined. Pulmonary capillaries were engorged with polymorphonuclear leukocytes undergoing fragmentation 15 minutes after endotoxin or E. coli organism injections. The endothelial cytoplasm contained large vacuoles and many vesicles, but there was no evidence of actual rupture of the cytoplasmic membranes. Endothelial cellular membranes appeared fuzzy and indistinct at sites where polymorphonuclear leukocytes were adhering. Fragmentation and loss of specific granules in polymorphonuclear leukocytes were noted one hour post-injection. The loss of specific granules, fragmentation of polymorphonuclear leukocytes, and focal areas of pulmonary edema were observed four hours following endotoxin or live E. coli organism injection. In markedcontrast to a previous report in the primate administered endotoxin, this investigation revealed no evidence for intravascular coagulation of fibrin and platelet aggregates. The widespread morphological alterations could explain some of the functional derangements previously observed in monkeys in shock.

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LEGENDS

Figure 1 - Femoral arterial pressure plotted as present change after Infusion of endotoxin or E. coli. The final point in each line represents the time of sacrifice.

Figure 2 - Endotoxin, 15 minute specimen. Rounding of the alveolar spaces and focal areas of pulmonary edema are observed. The capillaries are ectatic and are filled with polymorphonuclear leukocytes. H & E 170 X

Figure 3 - Endotoxin, 4 hour specimen. Extensive atelectasis is noted throughout field. The alveolar walls are very cellular and the predominant cell type is the poly. H & E 170 X

Figure 4 - Endotoxin, 15 minute specimen. Free lamellar material is seen within the alveolar spaces (A). The pulmonary capillaries are engorged with polymorphonuclear leukocytes (L), which are de-granulating and rupturing (arrow). The endothelium is contin-uous but is fuzzy in those areas of leukocytic sticking (double arrows). The perivascular space is edematous (P) and the reticu-lar and collagen fibers are separated. UA + LC 6460 X

Figure 5 - Endotoxin, 15 minute specimen. The normal alveolar type I cell (AT II) projects into the alveolar space (A). The alveolar type I epithelium (AT I) is edematous. The perivascular space (P) is markedly edematous with the collagen and reticular fibers separated. The leukocyte (L) is undergoing fragmentation (arrow). The endothelium shows no evidence of discontinuity. UA + LC 9000 X

Figure 6 - Endotoxin, 1 hour specimen. Free lamellar material is noted within the alveolar spaces (A). The capillary is ectatic and

filled with cellular remnants, degenerating leukocytes (L),
and specific granules. The endothelium is intact, however
large vacuoles are seen with the cytoplasm (arrows). UA + LC
5610 X

Figure 7 - Endotoxin, 1 hour specimen. The endothelial cytoplasmic vacu-
oles are seen (arrows). The leukocytes (L) show a varied con-
tent of granules. The cellular membranes of several of the
leukocytes are fuzzy and indistinct. UA + LC 11,700 X

Figure 8 - Live organisms, 1 hour specimen. The leukocytes (L) appear to
be fused to the endothelium at several different sites (arrows).
Some endothelial perinuclear vacuoles are seen (double arrows).
The leukocytes show a loss of cytoplasmic specific granules.
UA + LC   12,470 X

Figure 9 - Live organisms, 4 hour specimen. The alveolar type I epithelium
(ATI) is focally edematous. Within the alveolar spaces (A),
traces of plasma transudate can be seen. In some areas, the
perivascular space (P) is somewhat widened by fluid. The
capillari   (C) contain leukocytes (L) and cellular debris.
The cytoplasm of the leukocytes is quite granular, and a loss
of the specific granules is noted in several cells.
UA + LC 5,610 X

Figure 10- Live organisms, 4 hour specimen. One of the leukocytes (L)
has an ingested bacillus (B). Varied degrees of degranulation
are evident within the leukocytic cytoplasm. The perivascular
space (P) is edematous, however, the alveolar spaces (A) are
free from pulmonary edema. UA + LC 7,590 X

Figure 11 - Live organisms, 4 hour specimen. Within the capillary lumen (C),

two polys (L) are seen, both of which are markedly degranulated. The cytoplasm contains many granules of glycogen. The polys are attached to several areas of the endothelium and distinct cellular outlines are fuzzy (arrow). The perivascular space is edematous (P). US + LC 18,600 X

Figure 12- Endotoxin, 4 hour specimen. Within the capillary lumen, many free granules are evident. The leukocytes (L) and platelets show an abundance of glycogen granules. The cellular membranes of the leukocytes are indistinct. At several sites, the cells are sticking to the endothelium (arrows). At the double arrow, is a cytoplasmic portion of a poly which appears to be starting to emigrate, a rare observation. UA + LC 19,220 X

Figure 13- Endotoxin, 4 hour specimen. Perivascular space edema (P) is demonstrated. Lamellar and cellular remnants are seen within the alveolar space (A). A large membrane bound component containing granular material (M) is seen within the capillary lumen. The leukocyte is sticking to the endothelium at one point (arrow). UA + LC 10,200 X

Figure 14- Endotoxin, 4 hour specimen. Within the capillary (C), free granules and cytoplasmic remnants are noted. A degranulated poly (N), with abundant cytoplasmic glycogen, has several ruptures within its cellular membrane. The endothelium is attenuated but intact. UA + LC 18,600 X
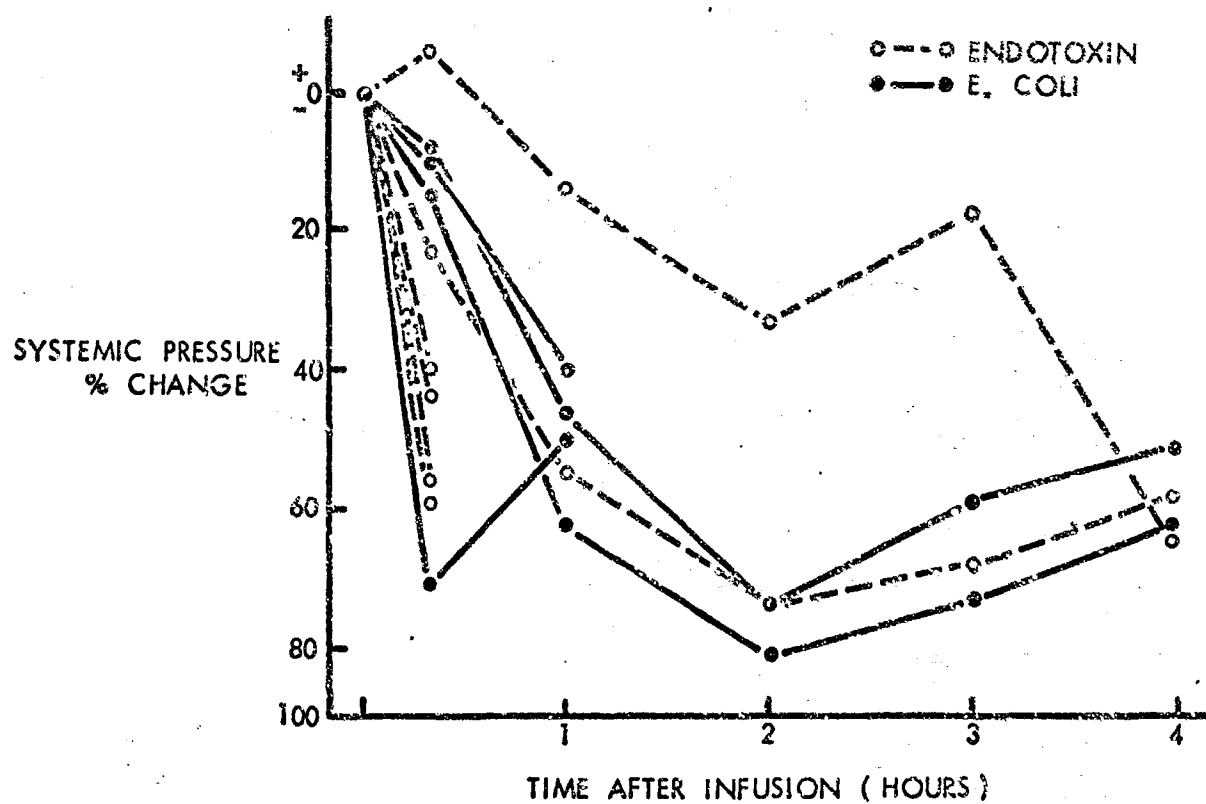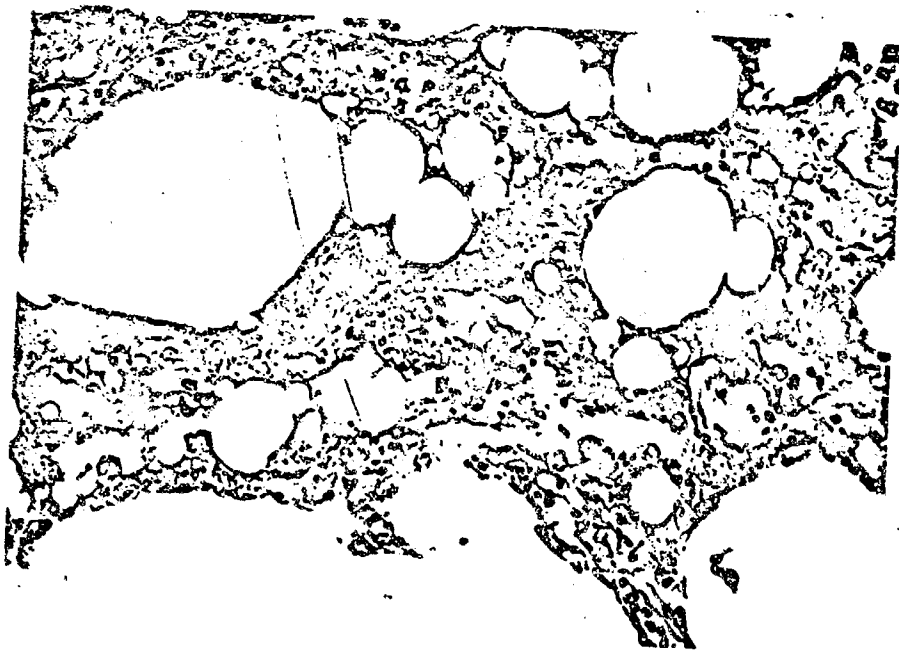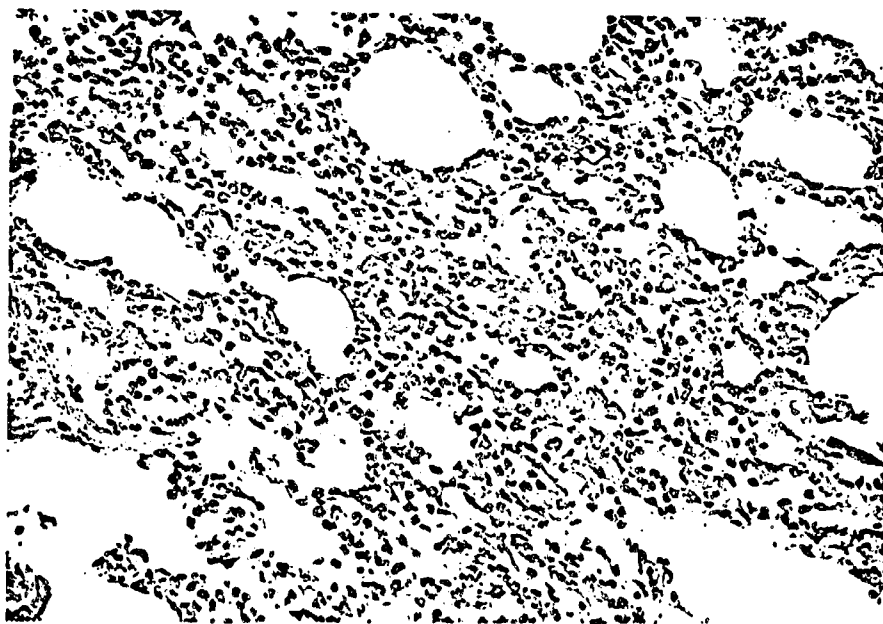
FIGURE 1

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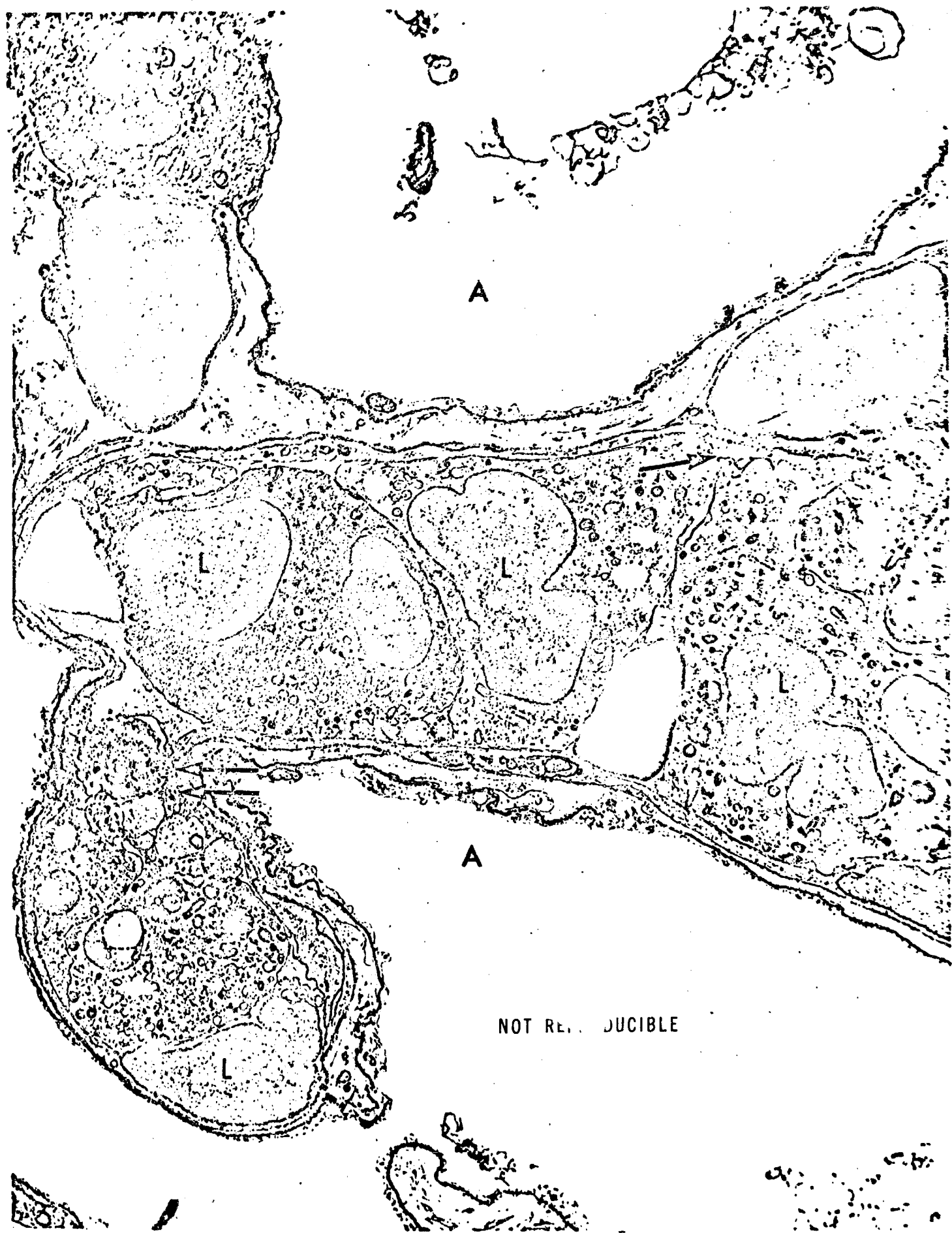
FIGURE 2

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FIGURE 3

FIGURE 4

FIGURE 5

A
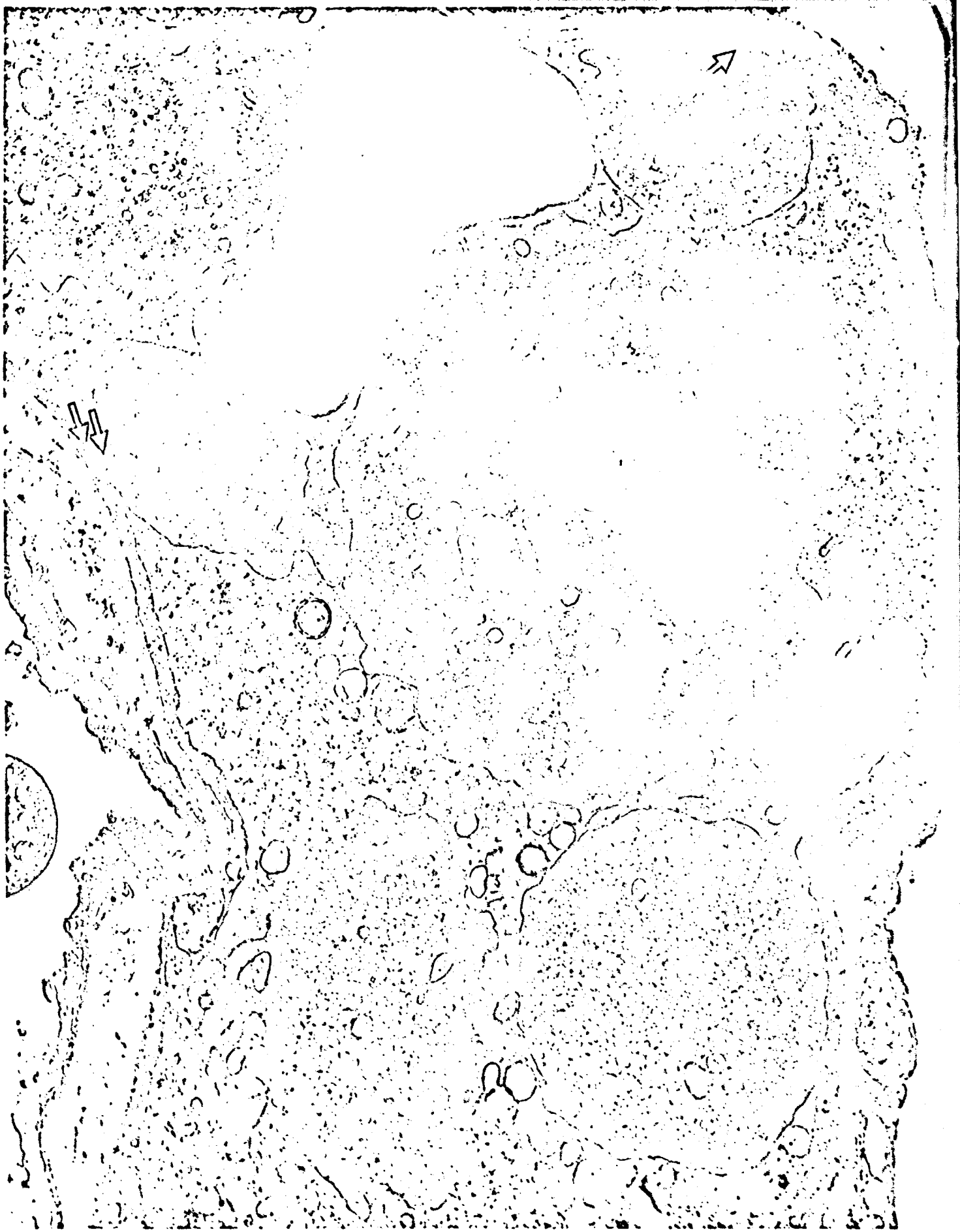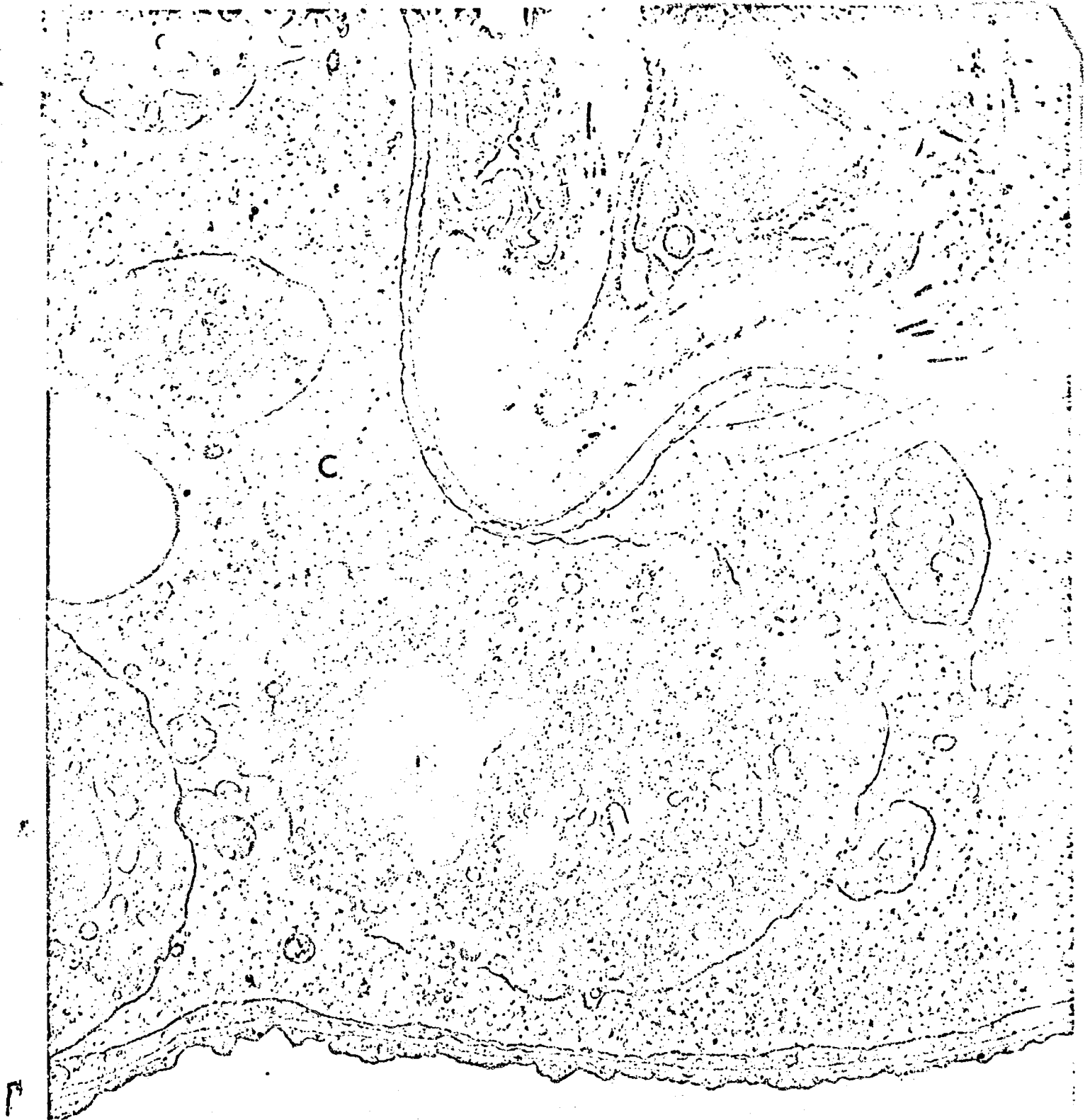
FIGURE 6

FIGURE 7

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FIGURE 9

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FIGURE 11

FIGURE 12

FIGURE 13

FIGURE 14

DOCUMENT CONTROL DATA - R & D

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13. ABSTRACT

The major purpose of the current study was to extend and re-evaluate the previous investigation on the primate administered endotoxin. Waisbren (1964) recently criticized the animal shock model in which endotoxin was used to simulate septic shock in man. Pulmonary functional abnormalities reported in priamtes administered live E. coli organisms have included among other changes, hyperventilation, increased physiological dead space, increased alveolo-arterial oxygen gradients, decreased compliance of the lungs and increased surface tension of lung extracts. The ultrastructural morphological effects of live organisms in the lung of the primate have not been previously described. The second purpose of the present investigation was to utilize an animal model more closely approximating the clinical entity of septic shock by including a series of experiments with intravenously injected live E. coli organisms.

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